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Wound infection and tissue damage are common and sever complications of injuries and burns sustained in battle. The surgical excision of dead and devitalized tissue, antibiotics, and the removal of foreign matter are the mainstay of current therapies. Prevailing treatment strategies are restricted, however, by the inability to initiate effective countermeasures until removal from the uncontrolled environment of the field of battle. This results in high rates of infections and serious medical complications. Mammalian secretory fluids maintain antimicrobial activity as part of host defenses against invading bacteria, parasites, and viruses. Among the agents that provide antimicrobial protection in these fluids are the copper-containing protein ceruloplasmin, and the leukocyte-derived hemoprotein enzymes myeloperoxidase and eosinophil peroxidase. Peroxidases and ceruloplasmin interact with microbes and may serve as Enzymatic Wound Disinfectants by catalyzing the formation of reactive oxidants and diffusible radical species that inflict oxidative damage upon invading parasites and pathogens. We propose to develop and test Enzymatic Wound Disinfectants as a practical, rapid, and effective treatment for wounds and burns. Molecular biological efforts will focus on the cloning, expression, isolation, and engineering of thermostable, protease-resistant peroxidases and ceruloplasmin. Biochemical and physiological initiatives will focus on characterizing enzymatic activity and stability, mechanisms of action, and clinical utility.

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Introduction

Wound infection and tissue damage are common and sever complications of injuries and burns sustained in battle. The surgical excision of dead and devitalized tissue, antibiotics, and the removal of foreign matter are the mainstay of current therapies. Prevailing treatment strategies are restricted, however, by the inability to initiate effective countermeasures until removal from the uncontrolled environment of the field of battle. This results in high rates of infections and serious medical complications.

Mammalian secretory fluids maintain antimicrobial activity as part of host defenses against invading bacteria, parasites, and viruses. Among the agents that provide antimicrobial protection in these fluids are the copper-containing protein ceruloplasmin, and the leukocyte-derived hemoprotein enzymes myeloperoxidase and eosinophil peroxidase (1-4). Peroxidases and ceruloplasmin (5,6) interact with microbes and may serve as Enzymatic Wound Disinfectants by catalyzing the formation of reactive oxidants and diffusible radical species that inflict oxidative damage upon invading parasites and pathogens.

We propose to develop and test Enzymatic Wound Disinfectants as a practical, rapid, and effective treatment for wounds and burns. Molecular biological efforts will focus on the cloning, expression, isolation, and engineering of thermostable, protease-resistant peroxidases and ceruloplasmin. Biochemical and physiological initiatives will focus on characterizing enzymatic activity and stability, mechanisms of action, and clinical utility.

Body (progress report)

Task 1. To express tagged mature and fully processed recombinant human myeloperoxidase.

a. isolate cDNA, add His tag, and place in appropriate vector, determine optimal transfection conditions and select stable transfectants

Isolation of MPO cDNA:

During the initial phase of the study, human myeloperoxidase cDNA was isolated by PCR amplification from a human leukocyte cDNA library. Oligonucleotide primers were designed complementary to the 5' and 3' termini of the MPO gene thus spanning the full-length coding sequence of MPO cDNA and these were used to amplify MPO cDNA from the human leukocyte cDNA library. PCR amplification was performed using a proofreading DNA polymerase (Pfu). The primers were designed to include convenient restriction enzyme sequences to allow for sub-cloning into the pUC18 vector. Upon gel purification and isolation of the amplified MPO cDNA and subsequent construction of this vector, competent E. coli bacteria was transformed and grown in order to obtain milligram amounts of purified DNA for subsequent genetic manipulations.

Vector selection and 6x histidine-tagging:

For the purpose of protein purification by nickel chromatography, we positioned a six repeat histidine tag at the 3' end of the MPO gene. This was achieved by PCR amplification of the full

length MPO cDNA. Oligonucleotide primers were designed to re-engineer the MPO coding sequence prior to sub-cloning into the pcDNA3.1/V5-His TOPO vector (Invitrogen). This necessitated removal of the 3' TAG stop codon to allow for translational read-through to the in-frame histidine repeat sequence, situated approximately 20 amino acids downstream from the terminal MPO amino acid, serine. The pcDNA3.1/V5-His TOPO vector was selected for expressing MPO in eukaryotic cells due to the strong cytomegalovirus (CMV) promoter and the positioning of a of six histidine residue encoding tag in frame with the MPO open reading frame at the 3' end. This vector also contains a convenient selection marker, conferring resistance (via the neo^R gene) of positively transfected eukaryotic host cells to the drug G418.

Subcloning and sequence confirmation:

The amplified MPO cDNA lacking the stop codon was subcloned into the pcDNA3.1 His TOPO vector according to the manufacturer's instructions that utilizes the ligation activity associated with the enzyme topoisomerase. Following each round of PCR amplification, the amplified regions were sequenced from a set of internal primers positioned at approximately 400 base pair intervals spanning the entire length of the MPO gene and compared to the MPO DNA sequence provided within the Genbank Accession number. NM 000250 to insure that no unintended genetic modifications had been introduced during PCR amplification.

Optimization of transfection conditions: Lipid mediated transfection (lipofectamine), and electroporation:

We have successfully introduced the MPO expressing plasmid into eukaryotic cells (K562, monocyte precursors derived from leukocytes of a patient with chronic myelogenous leukemia) by lipid mediated transfection according to the manufacturer's instructions (lipofectamine, Invitrogen) and by electroporation using the current parameters of 960 μF and voltage 230mV with time constants on average of 46 milliseconds. Initially these were employed in transient transfection experiments to examine the efficiency and feasibility of MPO expression as assayed by standard peroxidase assays using the substrates guaiacol and 3,3',5,5' tetramethylbenzadine (TMB) and by immunoblotting to examine protein maturation and proteolytic processing.

Method to select for high copy number integrants and MPO expressing clones:co-transfection with GFP-expressing plasmid:

As we are interested to maximize expression of MPO and the enzymatic activity to be recovered, we endeavored to create a transfection method that would allow us to immediately select for those cells transfected with maximum copies of MPO cDNA. To accomplish this, we co-electroporated a second plasmid encoding the green fluorescent protein (GFP) together with the His-tagged MPO vector in a 60-fold molar excess of MPO plasmid to GFP plasmid. In this fashion we made the reasonable assumption that all of the GFP expressing cells would also have taken up a corresponding ratio of MPO DNA. We then employed flow cytometric fluorescence activated cell sorting (FACS) to select for a population of cells expressing the highest levels of GFP (those falling within the fourth decade of the logarithmic scatter plot). This population was subsequently diluted to yield approximately one cell per well in 96-well microtiter plates, selected against G418, expanded and analyzed after one month for protein content and enzyme activity. To further boost enzyme activity levels during the course of stable transformant selection, the growth media was supplemented with 2 μM hemin, which has been reported to increase enzyme maturation and processing by 20% (7).

b. use antibodies to MPO heavy chain and His 6x tag to determine degree of maturation/proteolytic processing and retention of His tag, and if needed, re-engineer His tag.

Recombinant MPO protein in His-tagged, MPO-expressing cell-lines exhibits insufficient enzymatic activity:

Immunofluorescent microscopy experiments using an anti-MPO polyclonal antibody together with a fluorochrome conjugated secondary antibody revealed that MPO is expressed and correctly localized to the cytoplasm of transfected K562 cells. This pattern was identical to the subcellular distribution seen in endogenous MPO-expressing promyelocytic leukemia HL60 cells, used as a positive control. However, immunoblotting showed incomplete proteolytic processing of the precursor MPO protein into mature enzyme (heavy and light chain). Likewise, peroxidase activity assays showed no significant increase in enzyme activity between cell lysates prepared from transfected cells versus the parental line K562.

Re-engineering of His-tag due to proteolytic processing at 3'-end:

Lack of enzymatic activity coupled with a report in the literature suggesting that during the course of enzyme maturation, the terminal serine is proteolytically processed, led us to re-position the Histidine tag immediately upstream of the penultimate MPO amino acid residue alanine. An oligonucleotide primer was designed to contain this genetic modification which was then incorporated into the MPO construct by PCR-mediated amplification. The resulting construct was again sequenced to insure the fidelity of the MPO coding sequences.

Following transfection, a subsequent round of selection, and cell lysate preparation from isolated clonal cell populations, again we were unable to detect significant peroxidase activity levels. Immunoblotting again revealed that the MPO precursor protein was inefficiently processed. Additionally, we were unable to detect MPO protein via immunoblotting with an antibody directed against the histidine tag despite the fact that protein was abundant as seen via blotting with an anti-MPO antibody.

Expression of non-His-tagged, wild-type recombinant MPO:

Based upon our inability to recover peroxidase activity or efficient enzyme maturation in K562 cells expressing the histidine-tagged myeloperoxidase construct, we introduced a non-his tagged, wild-type MPO cDNA from the pcDNA3.1 vector (Invitrogen), which contains the same promoter and resistance markers as the pcDNA3.1/V5-His TOPO vector. While this strategy will not permit us to employ nickel column chromatography as we had planned, standard protocols exist in the scientific literature describing the rapid and efficient purification of myeloperoxidase from both tissue and tissue culture cell lines. Transfection and selection conditions were maintained as described above.

Selection of high expressing clones based upon peroxidase levels of activity:

We have screened over 200 G418-resistant clonal cell populations by peroxidase activity assays and we now have stocks of multiple clonal cell lines, established in the K562 parental line. These clonal populations have been selected based upon their high levels of enzymatic activity as assayed via guaicol and TMB and their high degree of proteolytic processing into the mature protein, as assessed by immunoblotting.

c. Purification and characterization, including kinetics, substrate selectivity, MALDI TOF mass spectrometry to determine size and estimation of carbohydrate content of recombinant enzyme, protease resistance and heat liability.

This work is in progress. See below.

Task 2. To express tagged mature and fully processed recombinant human eosinophil peroxidase.

Isolation of EPO cDNA:

During the initial phase of the study, human eosinophil peroxidase cDNA was isolated by PCR amplification from a human leukocyte cDNA library. Oligonucleotide primers were designed complementary to the 5' and 3' termini of the EPO gene thus spanning the full length coding sequence of EPO cDNA and these were used to amplify EPO cDNA from the human leukocyte cDNA library. PCR amplification was performed using a proofreading DNA polymerase (Pfu). The primers were designed to include convenient restriction enzyme sequences to allow for subcloning into the pUC18 vector. Upon gel purification and isolation of the amplified EPO cDNA and subsequent construction of this vector, competent E. coli bacteria (strain DH5a) was transformed and grown in order to obtain milligram amounts of purified DNA for subsequent genetic manipulations.

Vector selection and 6x-histidine tagging:

We positioned the six repeat histidine tag at the 3' end of the EPO gene according to a similar protocol used for tagging the MPO cDNA. This was achieved by PCR amplification of the full length EPO cDNA. Oligonucleotide primers were designed to re-engineer the EPO coding sequence prior to subcloning into the pCDNA3.1/V5-His TOPO vector (Invitrogen). This necessitated removal of the 3' stop codon to allow for translational read-through to the in-frame histidine repeat sequence, situated approximately 20 amino acids downstream from the terminal EPO amino acid, threonine. The pCDNA3.1/V5-His TOPO vector was selected for expressing EPO in eukaryotic cells due to the same features as described previously for MPO.

Subcloning and sequence confirmation:

The amplified EPO cDNA lacking the stop codon was subcloned into the pCDNA3.1 His TOPO vector as described for MPO. Following each round of PCR amplification, the amplified regions were sequenced from a set of internal primers positioned at approximately 400 base pair intervals spanning the entire length of the EPO gene and compared to the EPO DNA sequence provided within the Genbank Accession number. X14346 to insure genetic fidelity.

Optimization of transfection conditions: Lipid mediated transfection (Lipofectamine), and electroporation:

We have successfully introduced the EPO expressing plasmid into eukaryotic cells (K562, monocyte precursors derived from leukocytes of a patient with chronic myelogenous leukemia), known to support all of the post-translational modifications required for mature, catalytically active enzyme. As described for MPO, this was achieved both by lipid mediated transfection and by electroporation. Again, transient transfection experiments examined the efficiency and

feasibility of EPO expression as assayed by the standard peroxidase assays (guaiacol and 3,3',5,5' tetramethylbenzadine) and by immunoblotting to examine protein maturation and proteolytic processing.

Method to select for high copy number integrants and EPO expressing clones:co-transfection with GFP-expressing plasmid:

To maximize expression of EPO we used the GFP co-transfection method described for MPO that would allow for immediate selection of cells transfected with maximum copies of EPO cDNA. Positively transfected cells, measured by the intensity of their green fluorescence, were subsequently diluted to yield approximately one cell per well in 96-well microtiter plates, selected against G418, expanded and analyzed after one month for protein content and enzyme activity.

b. Use antibodies to MPO heavy chain and His 6x tag to determine degree of maturation/proteol ytic processing and retention of His tag, and if needed, re-engineer His tag.

Recombinant EPO protein in His-tagged EPO-expressing cell-lines exhibits enzymatic activity:

Immunoblotting experiments using an anti-EPO monoclonal antibody directed against EPO heavy chain showed incomplete proteolytic processing of the precursor MPO protein into mature enzyme (heavy and light chain). Despite this inefficient processing, peroxidase activity assays showed a significant enzyme activity in transfected cell lysate.

Removal of EPO His tag due to inaccessibility in cell lysate:

As seen for recombinant MPO, we were unable to detect EPO protein via immunoblotting with an antibody directed against the histidine tag despite the fact that protein was abundant as seen via blotting with an anti-EPO antibody and that transfected cell lysates exhibited peroxidase activity . While proteolytic processing at the carboxy terminus has not been reported for EPO, this data suggested that similar to recombinant MPO, the histidine tag was being removed or was not accessible to antibody recognition (and therefore Nickel-affinity purification). In light of our expression studies with MPO, we elected to forgo expression of the histidine tagged-EPO and proceeded to transfect and re-select for K562 cells that express non-his tagged EPO cDNA.

Selection of high expressing clones based upon peroxidase levels of activity:

We are currently screening G418-resistant clonal cell populations by peroxidase activity assays and we now have stocks of multiple clonal cell lines, established in the K562 parental line. These clonal populations have been selected based upon their high levels of enzymatic activity as assayed via guaicol and TMB and their high degree of proteolytic processing into the mature protein, as assessed by immunoblotting.

c. Purification and characterization, including kinetics, substrate selectivity, MALDI TOF mass spectrometry to determine size and estimation of carbohydrate content of recombinant enzyme, protease resistance and heat liability.

This work is still in progress. See below.

Task 3. To express tagged mature and fully processed recombinant human ceruloplasmin.

Stable transfection of COS-7 cells with a full-length Cp cDNA construct:

We have initiated experiments to determine optimal conditions for stable expression of recombinant Cp in COS-7 cells. We have extended the methods previously used by us in transient transfection of Cp in these cells (8). We also have taken advantage of the new information gained from our series of experiments on expression of MPO (above). We first tested the susceptibility of COS-7 cells to G418 to determine the optimal concentration of the selection agent. We used a construct containing the full-length human Cp cDNA, lacking the 5'- and 3'-untranslated regions, in the pcDNA3 plasmid vector (pcDNA3-Cp). Sub-confluent cells were transfected with lipofectamine and a range of amounts of pcDNA3-Cp. During subsequent passages gradually increasing concentrations of G418 were applied to kill untransfected or weakly expressing cells. After multiple passages, two distinct pools of cells were selected that survived G418 selection pressure. Expression was tested by immunoblot analysis using highly specific rabbit anti-human Cp IgG. High-level expression of full-length protein was established. We are currently determining the activities of the recombinant protein, namely LDL oxidase and ferroxidase activities. To this end we have optimized the conditions for improving the accuracy and sensitivity of the ferroxidase assay. The major principle in this improved assay is that the fold-increase in ferroxidase activity of ceruloplasmin is considerably higher when the assay is conducted in hypoxic conditions (1% O2). We are also using this method to produce recombinant Cp with defined point mutations that individually block each of these activities.

Task 4. Develop large-scale purification methods for each of the recombinant enzymes.

Purification of MPO and EPO:

This work is still in progress. Our recombinant K562 suspension cells are grown in a FiberCell^{tm} 60 ml hollow fiber cartridge (Fiber Cell Systems) that yields approximately 2 x 10^9 cells per week (3-5 ml of packed cell pellet).

Our protocol is adapted from methods described to purify MPO from the promyelocytic cell line HL60 (9) and from extracted leukocytes (10). Briefly, cells are lysed in cetyltrimethylammonium bromide detergent (CTAB) followed by lectin-affinity chromatography and molecular filtration. It has been reported that MPO purified from 10 ml of HL60 cell pellet routinely yielded 12-20 mg of pure MPO.

We have estimated our level of MPO expression based upon our initial activity assays, on immunoblots of recombinant human MPO, and on estimation of protein concentration from recombinant MPO-expressing cell lysates compared with endogenous MPO from HL60 cells. We conclude that we can achieve expression levels of recombinant MPO in K562 cells which approach those of the promyelocytic HL60 cells.

Parallel studies employing stable transfectants of K562 cells with non-His-tagged EPO have established that we are able to produce enzyme with catalytic activity and appropriate proteolytic processing. Using published methods for purification of EPO from eosinophils (4),

we have been able to translate these methods into purification of EPO from cultured, transfected K562 cells. Efforts are currently underway for large scale preparation using the hollow fiber system.

In summary, the results obtained during the first year of the proposed research suggest that after initial technical impediments (inadequate enzymatic activity and inefficient protein maturation seen for recombinant protein expressed from the histidine-tagged construct) our results with the wild type MPO now suggest that we are now on track to produce high levels of mature, catalytically active recombinant enzymes for biochemical and kinetic characterization and ultimately for studies designed to examine their use as effective microbicidal reagents.

Task 5. Test in vivo efficacy of recombinant enzymatic wound disinfectants.

Role of Cp in bactericidal activity in vivo:

Previous reports of bactericidal activity of Cp are limited to in vitro experiments and studies using human plasma (11,12). We have initiated studies on the in vivo effect of Cp on bactericidal activity in mice. We have used the methods described in studies of MPO-deficient mice (13). We used the progeny of a cross of two heterozygous Cp1 mice (background is 50% 129/SV, 50% black Swiss-Webster). We use here the nomenclature in which the gene name is followed by the number of wild-type alleles, i.e., Cp0 is knock-out and Cp2 is wild-type. Two Cp0 and two Cp2 (all female) mice were used in this pilot study.

Candida albicans yeast (strain 820) were cultured in Sabouraud dextrose medium. The yeast were collected by centrifugation, washed in ice-cold sterile water and resuspended in normal saline. 6×10^8 colony-forming units of C. albicans were injected into the peritoneal cavity of each mouse. The Cp0 mice died on day 2 and day 3 after infection while the two Cp2 mice began normal activity by day 4 and recovered completely. These data, while very preliminary, are suggestive that Cp may have as important bactericidal activity in vivo. Experiments are under way in which greater numbers of mice in an in-bred background will be used.

Key research accomplishments

1. We have isolated the full-length cDNAs encoding the myeloperoxidase and eosinophil peroxidase enzymes.
2. The MPO and EPO cDNAs were subcloned into a eukaryotic cell expression vector containing an appropriate selection marker and successfully introduced into a monocyte-precursor cell line known to support all of the post-translational modifications necessary to achieve mature, catalytically active enzyme.
3. We successfully produced catalytically active, recombinant peroxidase enzyme in cells transiently transfected with these plasmids, as indicated by peroxidase activity assays using transfected cell lysates.

4. We developed a flow-cytometry based transfection protocol that allows us to select for clonal populations that express the highest amounts of recombinant peroxidase enzymes.

5. We have begun purifying mature, catalytically active recombinant MPO and EPO enzyme from a high-expressing clonal population of transfected K562 cells, selected based upon its peroxidase activity after screening more than 200 transfected clonal cell lines.

6. We have shown that the six-repeat histidine tag, inserted at the carboxyl terminus of MPO for the purpose of aiding in the enzyme recovery and purification protocol, sufficiently impeded peroxidase activity so as to warrant its removal from the recombinant enzyme. Due to the interference in enzyme activity brought about by the histidine tag, we have resumed expressing recombinant peroxidase containing the correct 3' terminus lacking the tag.

7. We have established conditions for stable transfection and high-level expression of Cp by COS-7 cells.

8. We have preliminary data indicating anti-bactericidal activity of native Cp in vivo.

Reportable outcomes

No manuscripts submitted to date.

Conclusions

The results obtained during the first year of the proposed research suggest that after initial technical impediments (inadequate enzymatic activity and inefficient protein maturation seen for recombinant protein expressed from the histidine tagged MPO construct) our results with the MPO, EPO, and Cp cDNAs suggest that we are now on track to produce high levels of fully mature, catalytically active recombinant enzymes for biochemical and kinetic characterization and ultimately for studies designed to examine their use as effective microbicidal reagents.

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Appendices

None